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Brain Transplantation of Immortalized Human Neural Stem Cells Promotes Functional Recovery in Mouse Intracerebral Hemorrhage Stroke Model

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Key Words. Human neural stem cell • Permanent cell line • Intracerebral hemorrhage • Stroke • Brain transplantation • Cell therapy

ABSTRACT

We have generated stable, immortalized cell lines of human NSCs from primary human fetal telencephalon cultures via a retroviral vector encoding v-*myc*. HB1.F3, one of the human NSC lines, expresses a normal human karyotype of 46, XX, and nestin, a cell type-specific marker for NSCs. F3 has the ability to proliferate continuously and differentiate into cells of neuronal and glial lineage. The HB1.F3 human NSC line was used for cell therapy in a mouse model of intracerebral hemorrhage (ICH) stroke. Experimental ICH was induced in adult mice by intrastriatal administration of bacterial collagenase; 1 week after surgery, the rats were randomly divided into two groups so as to receive intracerebrally either human NSCs labeled with β-galactosidase (n = 31) or phosphate-buffered saline (PBS) (n = 30). Transplanted NSCs were detected by 5-bromo-4-chloro-3-indolyl-β-D-galactoside histochemistry or double labeling with β-galactosidase (β-gal) and mitogen-activated protein (MAP)2, neurofilaments (both for neurons), or glial fibrillary acidic protein (GFAP) (for astrocytes). Behavior of the animals was evaluated for period up to 8 weeks using modified Rotarod tests and a limb placing test. Transplanted human NSCs were identified in the perihematomal areas and differentiated into neurons (β-gal/MAP2$^+$ and β-gal/NF$^+$) or astrocytes (β-gal/GFAP$^+$). The NSC-transplanted group showed markedly improved functional performance on the Rotarod test and limb placing after 2–8 weeks compared with the control PBS group (p < .001). These results indicate that the stable immortalized human NSCs are a valuable source of cells for cell replacement and gene transfer for the treatment of ICH and other human neurological disorders. STEM CELLS *2007;25: 1204–1212*

INTRODUCTION

NSCs have recently aroused a great deal of interest not only because of their importance in basic research of neural development but also for their broad potential for stem cell-based therapy in neurological diseases such as stroke, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and spinal cord injury [1–3]. The existence of immature multipotent stem cells has been identified in the embryonic and adult human brain and several groups have generated stable, perpetual NSC lines with capacity to continuously proliferate and multipotent to differentiate [4–8].

To this end, we have previously generated permanent cell lines of human NSCs with the ability to proliferate, generating a large number of clonally related progeny and retaining its multilineage potential over time [5, 8]. To assess the potential of these human NSCs as a cell source for cell replacement therapies, we transplanted the human NSCs into the vicinity of intracerebral hemorrhage lesion sites in mice. Intracerebral hemorrhage (ICH) is a major cause of mortality and morbidity, and 20% of ICH patients die within 3 days after the onset of disease [9]. ICH represents at least 15% of all stroke in the Western population and a considerably higher proportion, at 50%–60%, in the Asian populations [10]. ICH is a lethal stroke type, since mortality approaches 50%, and neurological disability in survivors is common. Because medical therapy against ICH such as mechanical removal of hematoma, prevention of edema formation by drugs, and reduction of intracranial pressure shows only limited effectiveness, an alternative approach is required [11, 12].

Previous studies have indicated that NSCs or neural progenitor cells engrafted in animal models of stroke survive and ameliorate neurological deficits in the animals [13–17], raising the possibility of therapeutic potential of NSCs for repair of damaged brain in ICH patients. Recent studies have also indicated that NSCs or neural progenitor cells isolated from fetal human brain can be propagated and then transplanted into the brain of animal models of stroke and can restore brain function [13, 17]. This approach, however, is not widely accepted for stroke patients because of the moral, religious, and logistic problems associated with the use of human fetal tissues. This difficulty can be circumvented by utilization of immortalized

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cell lines of human NSCs. The studies related to the cellular and molecular properties of human NSCs run into difficulty in obtaining sufficient numbers and homogeneous populations of human NSCs, and primary NSCs can be provided for only a limited time before they undergo senescence. Generation of sustainable human NSC clones is necessary to circumvent these problems.

Previously, we have isolated a clonal human neural stem cell line (HB1.F3) that had been immortalized by a retroviral vector encoding the v-*myc* oncogene [5, 8], and this stable human cell line shows multipotent capacity to differentiate into neurons and glial cells [8, 18] and ameliorate neurological deficits in animal models of stroke [14–16], Parkinson's disease [19], Huntington's disease [20, 21], and lysosomal storage disease [22] following their transplantation into the brain. In the present study, after transplantation into the brain of ICH mice, transplanted human NSCs were found to migrate extensively from the site of implantation into other anatomical sites and to differentiate into neurons and glial cells and improve functional deficits. These results indicate that the HB1.F3 human NSCs should be a great value as a cellular source for the cell replacement and gene transfer for the treatment of human neurological disorders.

Materials and Methods

Primary Human Telencephalon Culture

Telencephalon tissue from a 15-week gestational embryo was dissociated into single cells by incubation for 40 minutes at 37°C in phosphate-buffered saline (PBS) containing 0.25% trypsin and 40 μg/ml DNase type 1, following the procedure previously described [23, 24]. A suspension of dissociated cells (10^6 cells/ml) in culture medium was plated on poly-L-lysine-coated six-well plates. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 5% fetal bovine serum, 20 μg/ml gentamicin, and 5 μg/ml amphotericin B, and medium was changed twice a week. Primary cultures of fetal telencephalon grown for 2–4 weeks in vitro consisted of neurons, astrocytes, oligodendrocytes, microglia, and NSCs and were used for gene transfer experiments. The permission to use the fetal tissues was granted by the Clinical Research Screening Committee involving Human Subjects of the University of British Columbia, and the fetal tissues were obtained from the Anatomical Pathology Department of Vancouver General Hospital.

Generation of Immortalized Human Neural Stem Cells

An amphotropic replication-incompetent retroviral vector encoding *v-myc* (transcribed from the retrovirus long terminal repeat plus neo transcribed from an internal SV40 early promoter), pLSN.v-myc, was used to infect human telencephalon cells (Fig. 1). This vector was generated using the ecotropic retroviral vector encoding v-myc, similar to that described for generating human NSC lines previously [8, 25, 26]. The PA317 amphotropic packaging cell line was infected with the pLSN.v-myc vector, and successful infectants were selected and expanded. Supernatants from the PA317 producer cell line (PASK1.2) contained replication-incompetent retroviral particles bearing an amphotropic envelope, which efficiently infected the human NSCs as indicated by G-418 resistance.

Two milliliters of culture supernatant (4×10^5 colony-forming units) from the packaging cell line and 8 μg/ml polybrene were added to telencephalon cells in six-well plates and incubated for 4 hours at 37°C. The medium was then replaced with fresh growth medium; infection was repeated 24 and 48 hours later. Seventy-two hours following the third infection, infected cells were selected with G-418 (200 μg/ml) for 12–14 days, and large clusters of cells were individually isolated and grown in poly-L-lysine-coated dishes. Individual clones were selected again by limited dilution and propagated further. At this phase of isolation, six individual clones were designated as HB1 NSC lines. One of these clones, HB1.F3, was used in the present study.

To provide an unambiguous identification of externally introduced human NSCs in the mouse brain, the F3 NSC line was infected with a replication incompetent retroviral vector encoding β-galactosidase (LacZ) and puromycin-resistant genes (F3.LacZ lines). NSCs were grown in a serum-free medium (DM4) consisting of DMEM with high glucose containing 10 μg/ml insulin, 10 μg/ml transferrin, 30 nM sodium selenate, 50 nM hydrocortisone, 0.3 nM triiodothyronine, and 20 μg/ml gentamicin [27]. Recombinant human basic fibroblast growth factor (bFGF, 10 ng/ml; PeproTech, Rocky Hill, NJ, http://www.peprotech.com/) was supplemented to the DM4 during the routine feeding. All chemicals except bFGF were obtained from Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com.

RT-PCR Analysis

HB1.F3 human NSCs were collected by centrifugation and total RNA was isolated using Trizol, according to manufacturer's protocol (Promega, Madison, WI, http://www.promega.com). One microgram of total RNA was reverse-transcribed into first-strand cDNA using oligo-dT primer. Reverse transcription was performed with avian myeloblastosis virus reverse transcriptase (Takara, Otsu, Japan, http://www.takara.co.jp/english) for 1 hour at 42°C, inactivated for 10 minutes at 95°C, and cooled to 4°C. The cDNA was diluted to a final volume of 25 μl, and a 2-μl aliquot was used in a polymerase chain reaction (PCR) containing 1× DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside-5'-triphosphate, 10 pmol of primers, and 2.5 units of Taq polymerase (Takara). The cDNA was amplified using 30 PCR cycles, and reverse transcriptase-polymerase chain reaction (RT-PCR) products were separated electrophoretically on 1.2% agarose gel containing ethidium bromide and visualized under UV light. The primers used for the RT-PCR for nestin, neurofilament low-molecular weight protein (NF-L), neurofilament medium-molecular weight protein (NF-M), neurofilament high-molecular weight protein (NF-H), glial fibrillary acidic protein (GFAP), human myelin basic protein (MBP), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotropin 3 (NT-3), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, bFGF, vascular endothelial cell growth factor (VEGF), CXC-chemokine receptor (CXCR)4, vascular endothelial cell growth factor receptor (VEGFR)-1, VEGFR-2, c-kit, endothelial cell growth factor receptor (EGFR), and glyceraldehyde-3-phosphate dehydrogenase are listed in Table 1 [28, 29].

Immunocytochemistry for Cultures

Immunocytochemical demonstration of cell type-specific markers in F3 human NSCs was performed as described previously [23, 24]. F3 NSCs on poly-L-lysine-coated Aclar plastic coverslips (9 mm in diameter; SPL, Poncheon, Korea, http://www.ispl.co.kr) were grown in DM4 serum-free medium supplemented with bFGF for 2–3 days, rinsed in PBS, and fixed in ice-cold methanol for 10 minutes at −20°C. The cells were incubated with primary antibodies specific for nestin (1:200, rabbit polyclonal; kindly provided by Dr. K. Ikeda, Tokyo Metropolitan Psychiatry Institute, Tokyo), human mitochondria (1:400, mouse monoclonal; Chemicon, Temecula, CA, http://www.chemicon.com), NF-L (1:1,000, mouse monoclonal; Chemicon), NF-H (1:1,000, rabbit polyclonal; Chemicon), and GFAP (1:1,000, rabbit; DAKO, Glostrup, Denmark, http://www.dako.com) for 48 hours at 4°C, followed by Alexa Fluor 594-conjugated anti-mouse IgG for 1 hour at room temperature, and then counterstained with 4,6-diamidino-2-phenylindole (DAPI) (2 μg/ml; Sigma). For demonstration of oligodendrocytes, F3 NSCs on coverslips were fixed in 4% paraformaldehyde for 2 minutes, washed in PBS, incubated in anti-galactocerebroside antibody (1:4, mouse monoclonal; Kim Lab) for 48 hours at 4°C, followed by Alexa Fluor 594-conjugated anti-mouse IgG for 1 hour at room temperature, and then counterstained with DAPI (2 μg/ml; Sigma).

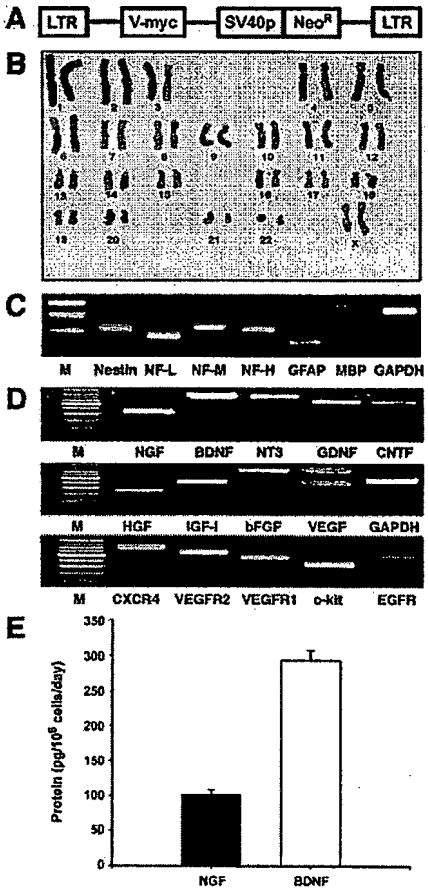

Figure 1. Generalization of immortalized human neural stem cell (NSC) line HB1.F3. **(A):** The retroviral vector encoding v-*myc* used in the present study in the generation of HB1.F3 human neural stem cell line. The vector has an internal SV40 promoter (SV40) from which NeoR is transcribed and translated. The *myc* oncogene (v-*myc*) is transcribed from the LTR. **(B):** Cytogenetic analysis indicates that HB1.F3 human neural stem cell line carries normal human karyotype of chromosomes, 22 pairs of autosomes, and two X chromosomes. **(C):** Gene expression of cell type-specific markers studied by reverse transcription-polymerase chain reaction (RT-PCR) in human HB1.F3 human neural stem cells. F3 cells express nestin (for neural stem cells), NF-L, NF-M, and NF-H (all for neurons), and GFAP (for astrocytes) but do not express MBP (for oligodendrocytes). **(D):** Gene expression of neurotrophic factors/growth factors and cellular receptors in HB1.F3 human neural stem cells studied by RT-PCR. F3 cells express NGF, BDNF, NT-3, GDNF, CNTF, HGF, IGF-1, bFGF, and VEGF and also receptors involved in cellular migration including CXCR4, VEGFR-1, VEGFR-2, c-kit, and EGFR. **(E):** HB1.F3 human neural stem cells constitutively secrete NGF and BDNF as shown by enzyme-linked immunosorbent assay quantification ($n = 3$). Abbreviations: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; CXCR, CXC-chemokine receptor; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; LTR, long terminal repeat; MBP, myelin basic protein; NeoR, neomycin-resistant gene; NF-H, high-molecular weight protein; NF-L, low-molecular weight protein; NF-M, medium-molecular weight protein; NGF, nerve growth factor; NT-3, neurotropin 3; SV40, SV40 promoter; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Table 1. Sequence of polymerase chain reaction primers

Gene	Sequence
Nestin	5′-CTCTGACCTGTCAGAAGAAT-3′ forward
	5′-GACGCTGACACTTACAGAAT-3′ reverse
NF-L	5′-TCCTACTACACCAGCCATGT-3′
	5′-TCCCCAGCACCTTCAACTTT-3′
NF-M	5′-TGGGAAATGGCTCGTCATTT-3′
	5′-CTTCATGGAAGCGGCCAATT-3′
NF-H	5′-CTGGACGCTGAGCTGAGGAA-3′
	5′-CAGTCACTTCTTCAGTCACT-3′
MBP	5′-ACACGGGCATCCTTGACTCCATCGG-3′
	5′-CCGGAACCAGGTGGTTTTCAGCG-3′
GFAP	5′-GCAGAGATGATGGAGCTCAATGACC-3′
	5′-GTTTCATCCTGGAGCTTCTGCCTCA-3′
NGF	5′-TCATCATCCCATCCCATCTTCCAC-3′
	5′-CACAGCCTTCCTGCTGAGCACAC-3′
BDNF	5′-ATGACCATCCTTTTCCTTACT-3′
	5′-CTATCTTCCCCTTTTAATGGT-3′
NT-3	5′-ATGTCCATCTTGTTTTATGTGA-3′
	5′-TCATGTTCTTCCGATTTTTC-3′
GDNF	5′-ATGAAGTTATGGGATGTCGT-3′
	5′-TTAGCGGAATGCTTTCTTAG-3′
CNTF	5′-ATGGCTTTCACAGAGCATT-3′
	5′-AACTGCTACATTTTCTTGTTGTT-3′
HGF	5′-AGGAGAAGGCTACAGGGGCAC-3′
	5′-TTTTTGCCATTCCCACGATAA-3′
IGF-1	5′-AAATCAGCAGTCTTCCAACCCA-3′
	5′-CTTCTGGGTCTTGGGCATGT-3′
bFGF	5′-GGGTGGAGATGTAGAAGATG-3′
	5′-TTTATACTGCCCAGTTCGTT-3′
VEGF	5′-GAAGTGGTGAAGTTCATGGATGTC-3′
	5′-CGATCGTTCTGTATCAGTCTTTCC-3′
CXCR4	5′-GTCATGGGTTACCAGAAGAA-3′
	5′-CAAGGAAAGCATAGAGGATG-3′
VEGFR2	5′-TATAGATGGTGTAACCCGGA-3′
	5′-TTTGTCACTGAGACAGCTTGG-3′
VEGFR1	5′-GTCACAGAAGAGGATGAAGGTGTCTA-3′
	5′-CACAGTCCGGCACGTAGGTGATT-3′
c-kit	5′-TATACAACCCTGGCATTATGTCC-3′
	5′-TGCGAAGGAGGCTAAACCTA-3′
EGFR	5′-TTCTCAGCAACATGTCGATGG-3′
	5′-TCGCACTTCTTACACTTGCG-3′
GAPDH	5′-CATGACCACAGTCCATGCCATCACT-3′
	5′-TGAGGTCCACCACCCTGTTGCTGTA-3′

Abbreviations: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; CXCR, CXC-chemokine receptor; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; MBP, myelin basic protein; NF-H, high-molecular weight protein; NF-L, low-molecular weight protein; NF-M, medium-molecular weight protein; NGF, nerve growth factor; NT-3, neurotropin 3; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

After wash in PBS, coverslips were mounted onto glass slides using gelvatol and examined under an Olympus laser confocal fluorescence microscope (Tokyo, http://www.olympus-global.com).

Cytogenetic Analysis

To establish whether the HB1.F3 cell line was diploid, the cells at various passages were subjected to metaphase mitotic arrest using colcemid, harvested by trypsinization, treated with hypotonic solution, fixed and stained by Giemsa solution, and then analyzed for polyploidy.

Mouse Model of ICH

Sixty-one male ICR mice weighing 25–35 g were used in the present study. All experimental procedures were approved by the

Animal Care Committee of the Ajou University School of Medicine. ICH was induced by stereotaxic, intrastriatal administration of bacterial collagenase by previously described methods [16, 30]. In brief, after an intraperitoneal injection of 1% ketamine (30 mg/kg) and xylazine hydrochloride (4 mg/kg), mice were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, http://www.kopfinstruments.com/). A burr hole was made, and a 30-gauge needle was inserted through the burr hole into the striatum (0.1 mm posterior, 4.0 mm ventral, and 2.0 mm lateral to the bregma); this needle was attached to a microinfusion syringe (fixed needle syringe, Hamilton 87942, Reno, NV, http://www.hamiltoncompany.com), and bacterial collagenase VII (0.075 U in 0.5-μl volume; Sigma) was injected into striatum at a rate of 0.25 μl/min for 2 minutes with an infusion pump (BAS Bioanalytical Systems, West Lafayette, IN, http://www.bioanalytical.com/), with the needle left in place for an additional 5 minutes after injection to avoid reflux. Animals were maintained in separate cages at room temperature with free access to food and water under a 12-hour light/dark cycle.

Cell Transplantation Procedure

To provide an unambiguous molecular tag for identification of the implanted F3 NSCs, the cell line was infected with a replication-incompetent retroviral vector encoding β-galactosidase (β-gal; LacZ) and puromycin-resistant genes, and a new subline F3.LacZ was generated. F3.LacZ human NSCs were dissociated into single cells by a brief trypsin treatment and suspended in PBS at 4×10^7 cells/0.1 ml and kept on ice until transplanted. Randomly selected ICH mice ($n = 31$) 1 week after ICH surgery received 0.5 μl (2×10^5 cells) of F3.LacZ cell suspension injected slowly for 5 minutes into overlying cortex of the hemorrhage lesion (0.1 mm posterior, 2.0 mm ventral, and 2.0 mm lateral to the bregma). The same volume of PBS was injected into the control group ($n = 30$). Immunosuppressant was not used in any of the animals.

Behavioral Testing

Behavioral testing was performed weekly with the Rotarod (Harvard Instrument, Holliston, MA, http://www.harvardapparatus.com) by two individuals blinded to mice treatment status [30]. In the Rotarod test, mice were placed on the Rotarod cylinder, and the time the animals remained on the Rotarod was recorded. The speed was slowly increased from 4 to 40 rpm within a period of 5 minutes. The trial was ended if the animal fell off the rungs or gripped the device and spun around for two consecutive revolutions. The animals were trained for 3 days before ICH operation. The maximum duration (in seconds) on the device was recorded with three Rotarod measurements 1 day before ICH induction. Motor test data are presented as percentages of the maximal duration compared with the internal baseline control (before ICH). The modified limb placing test is a version of a test previously described [31]. The test consists of two limb placing tasks that assess the sensorimotor integration of the forelimb and the hind limb by checking responses to tactile and proprioceptive stimulation. First, the rat is suspended 10 cm over a table, and the stretch of the forelimbs toward the table is observed and evaluated: normal stretch, 0 points; flexion with a delay (2-second) and/or incomplete, one point; and abnormal flexion, two points. Next, the rat is positioned along the edge of the table, with its forelimbs suspended over the edge and allowed to move freely. Each forelimb (forelimb, second task; hind limb, third task) is gently pulled down, and retrieval and placement are checked. Finally, the mouse is placed toward the table edge to check for lateral placement of the forelimb. The three tasks are scored in the following manner: normal performance, 0 points; performance with a delay (2-second) and/or incomplete, one point; and no performance, two points. A total of nine points means maximal neurological deficit, and 0 points means normal performance. In addition, the body weights of all animals were checked weekly for 8 weeks.

Histology and Immunohistochemistry

At the end of behavioral testing, each animal was anesthetized and perfused through the heart with 100 ml of ice-cold saline and 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were postfixed in the same fixative for 24 hours, followed with cryoprotection in 30% sucrose for 24 hours, and then 30-μm sections were prepared on a cryostat (Leica CM 1900; Leica, Heerbrugg, Switzerland, http://www.leica.com). Three sections through the needle entry site, which was identifiable on the brain surface, and sites 1.0 mm anterior and 1.0 mm posterior to the plane were processed for β-gal staining to analyze the hemisphere area. These sections are representative of the core of the ICH lesion. The morphometric analyses involved computer-assisted hand delineation of the area of the striatum, cerebral cortex, and ventricles, as well as the whole hemisphere. Adjacent serial coronal sections were processed for β-gal immunofluorescent staining with anti-β-gal antibody (1:1,000, mouse monoclonal; Sigma) to identify the β-gal/LacZ-positive transplanted cells. Antibodies specific for neurofilaments (NF-L+NF-M+NF-H for neurons, 1:100, mouse monoclonal; Zymed), MAP2 (1:1,000, mouse monoclonal; Chemicon), and GFAP (1:1,000, rabbit; DAKO) were used for cell type identification of neurons and astrocytes. Brain sections were incubated in primary antibodies overnight at 4°C as free-floating sections and followed by Alexa Fluor 594-conjugated anti-mouse IgG or Alexa Fluor 488-conjugated anti-mouse IgM (1:400; Molecular Probes) for 1 hour at room temperature. Negative control sections from each animal were prepared for immunohistochemical staining in an identical manner except the primary antibodies were omitted. Stained sections were then examined under an Olympus laser confocal fluorescence microscope.

Statistical Analysis

In all quantification procedures, two observers were blinded to the nature of the experimental manipulation. Statistical differences between two groups were determined by two-tailed Student's t-test.

RESULTS

Characterization of Immortalized Human Neural Stem Cells

The cloned HB1.F3 cells are tripolar or multipolar in morphology with 8–10 μm in size. Cytogenetic analysis of F3 NSCs showed a normal karyotype of human cells with a 46, XX, without any chromosomal abnormality as previously described (Fig. 1B). Results of RT-PCR analysis for mRNA isolated from HB1.F3 human NSCs grown in serum-containing medium (10% fetal bovine serum) are shown in Figure 1C and 1D. The transcript for nestin, a cell type-specific marker for NSCs and neural progenitor cells, and transcripts for NF-L, NF-M, and NF-H, cell type-specific markers for neurons, and the transcript for GFAP, a structural protein and a cell type-specific marker for astrocytes, are all expressed by NSCs and their progeny (Fig. 1C). However, the transcript for MBP, a structural protein and a specific cell type-specific marker for oligodendrocytes, was not demonstrated. These results indicate that F3 cells grown in serum-containing medium undergo asymmetrical division by which one daughter cell remains as NSCs and continues cell division while another one undergoes terminal differentiation into neurons or glial cells. Gene expression of neurotrophic factors/growth factors in F3 NSCs was studied by RT-PCR. The results demonstrate that the F3 NSCs express NGF, BDNF, NT-3, GDNF, CNTF, HGF, IGF-1, bFGF, and VEGF (Fig. 1D). In addition, F3 NSCs expressed specific receptors involved in cellular migration, including CXCR4, VEGFR-1, VEGFR-2, c-kit, and EGFR (Fig. 1D). Among the neurotrophic factors studied by the RT-PCR, we determined secretion of selected neurotrophic factors, NGF and BDNF, in F3 NSCs by enzyme-linked immunosorbent assay quantification, and the results in-

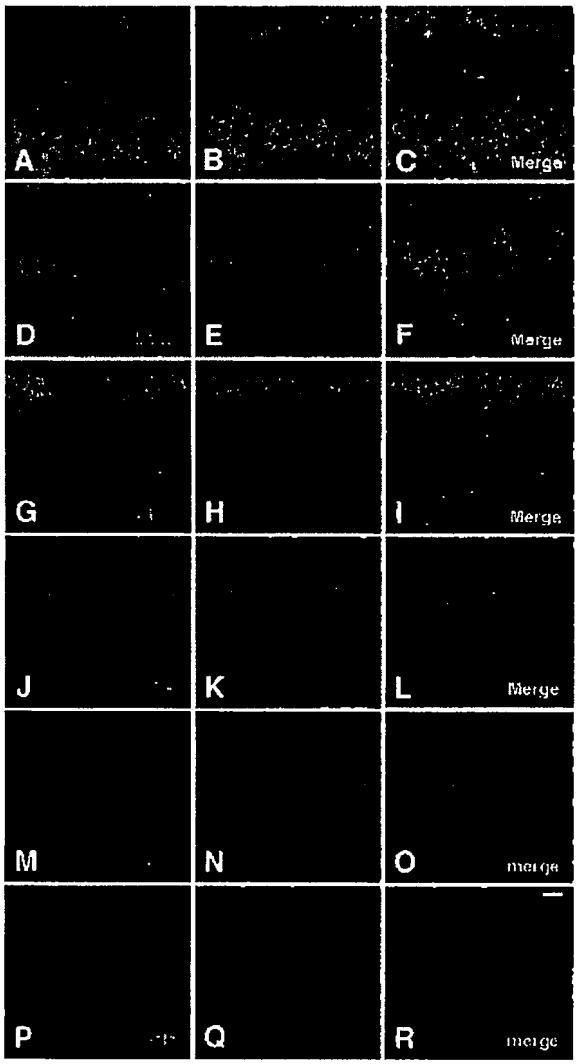

Figure 2. HB1.F3 human neural stem cells on coverslips were immunostained with anti-nestin (**A–C**), -human mitochondria (**D–F**), -NF-L (**G–I**), -NF-H (**J–L**), -GFAP (**M–O**), or -GalC (**P–R**) antibodies followed with Alexa Fluor 594 secondary antibody. The nuclei of F3 cells were detected with DAPI. Scale bar = 50 μm. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; Hmito, human mitochondria; NF, neurofilament.

dicate that the F3 NSCs constitutively secrete NGF and BDNF as high as 100 and 300 pg/10^6 cells/day, respectively (Fig. 1E).

When F3 NSCs were grown in DM4 serum-free medium supplemented with bFGF for 3–14 days and processed for immunostaining of nestin, a cell type-specific marker for NSCs, 100% of F3 cells were nestin-positive (Fig. 2A–2C) and also immunoreaction-positive for an antibody specific for human mitochondrial antigen (Fig. 2D–2F), indicating that F3 cells are indeed neural stem cells and are also of uniquely human origin as demonstrated earlier [8, 25]. To induce differentiated cell types in NSCs, F3 cells were switched to serum-containing medium, which consisted of DMEM supplemented with 10% fetal bovine serum but without bFGF. Immunochemical determination of cell type-specific markers for central nervous system (CNS) cell types was performed using antibodies specific

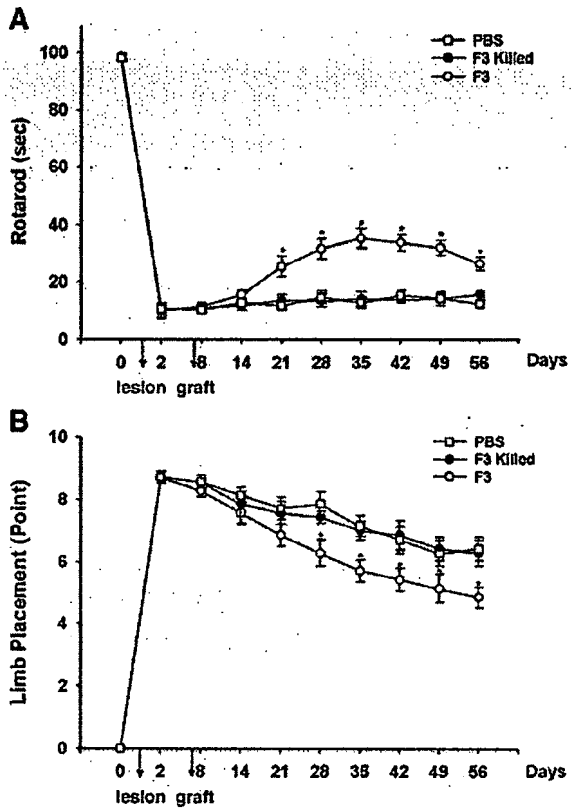

Figure 3. In the Rotarod test and limb placing test, the NSC-transplanted group showed a behavioral improvement from 21 days on, and these benefits continued up to 8 weeks ($p < .001$). Abbreviations: PBS, phosphate-buffered saline; sec, second(s).

for neurons, astrocytes, and oligodendrocytes. When F3 cells were grown in serum-containing medium, there was more than 40%–50% of total cells expressing low-molecular weight neurofilament proteins (68 kDa) (NF-L) (Fig. 2G–2I) and 10%–15% of cells expressing high-molecular weight neurofilament protein (200 kDa) (NF-H) (Fig. 2J–2L). In addition to neurons, smaller number of F3 cells grown in serum-containing medium expressed GFAP, a cell type-specific marker for astrocytes (>5%) (Fig. 2M–2O), whereas none of galactocerebroside-positive cells, a surface antigen-specific for oligodendrocytes, was found (Fig. 2P–2R). These results indicate that F3 human NSCs are pluripotent and capable of differentiation into neurons and astrocytes under stable culture conditions.

Human NSCs Improve Functional Deficits in Mice with Experimental ICH

The NSC-transplanted group showed better performance on the Rotarod test and limb placing test after 2 weeks compared with the control group receiving PBS instead of human NSCs (Fig. 3A, 3B; $p < .001$), and the effect persisted for up to 8 weeks. During the initial 2 weeks, the transplantation group showed a small improvement in the Rotarod test and limb placing. From the 2 weeks onward, the NSC-transplanted group showed better performance than the control group. The body weights of the two groups over the entire 8-week period were not different (data not shown).

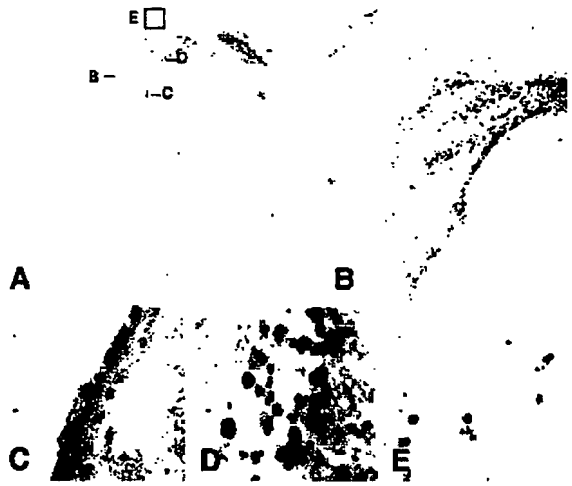

Figure 4. One week after an intracerebral hemorrhage lesion was produced by an intrastriatal injection of collagenase, LacZ (β-galactosidase)-labeled HB1.F3 human neural stem cells were transplanted in overlying cerebral cortex. Two weeks after the cell transplantation, LacZ-positive F3 cells were found to migrate into the hemorrhage core and surrounding brain sites. Bars = 0.5 mm (A), 200 μm (B), and 50 μm (C–E).

Transplanted Human NSCs Migrate, Survive, and Differentiate into Neurons and Astrocytes

Following transplantation into the ICH mouse cerebral cortex overlying hemorrhage lesion site, LacZ-positive human NSCs migrated selectively to the hemorrhage core and also located on the border of the lesion and further away from the injection sites (Fig. 4A–4E). A large population of the transplanted β-gal-positive cells (50%–60%) was also GFAP-positive and took the morphology of endogenous astrocytes (Fig. 5G–5I). Many of β-gal/GFAP double-positive cells were found along the border of hemorrhagic core. A smaller population (30%–40%) of transplanted cells differentiates into NF-positive (Fig. 5D–5F) or MAP2-positive (Fig. 5A–5C) neurons in the perihematomal sites. These results indicate that the majority of grafted F3 human NSCs differentiate into neurons and also astrocytes by responding to developmental signals provided by the locus of implantation.

Down-Regulation of v-*myc* Oncogene Expression

The F3 human NSC line has been generated via transduction with a retroviral vector encoding v-*myc* oncogene and transplantation of these cells in brain of experimental animals might result in tumorigenesis. However, there was no tumor growth in the brain in the ICH mice grafted with F3 human NSCs (data not shown). In the ICH mouse brain with grafted F3 cells, none of β-gal-positive F3 cells expressed pan-*myc* immunoreaction, indicating that down-regulation of v-*myc* occurs constitutively and spontaneously in v-*myc*-propagated human NSCs (Fig. 5J–5O). These observations suggest that v-*myc* is regulated by the normal developmental mechanisms in host animals that down-regulate endogenous cellular *myc* in CNS precursors during mitotic arrest and/or differentiation. The loss of v-*myc* expression in stably engrafted NSCs following transplantation is consistent with the invariant absence of neoplasm formation derived

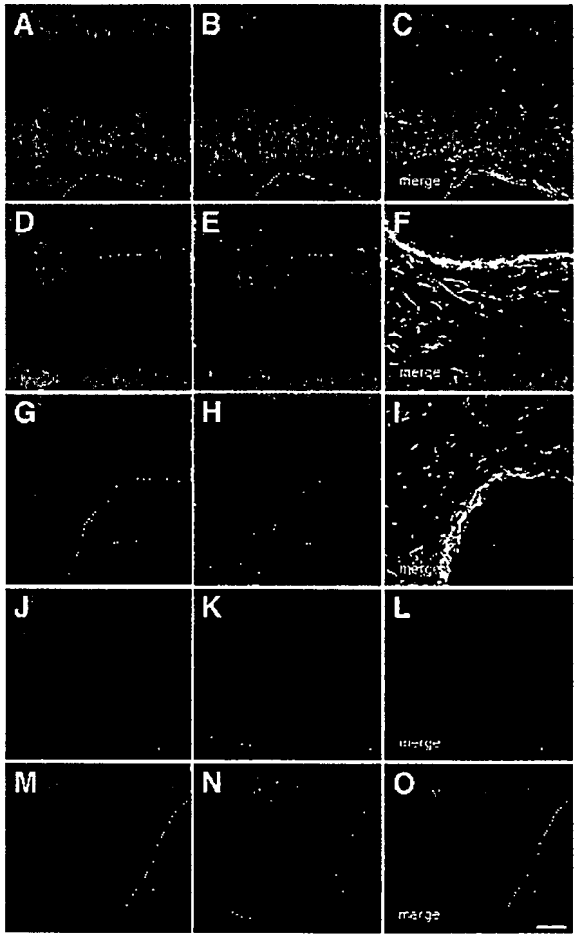

Figure 5. β-Galactosidase-labeled HB1.F3 human neural stem cells in the lesion sites are found to differentiate into neurons as shown by neuron specific markers MAP2 (**A–C**) and neurofilament proteins/NF (**D–F**) or into astrocytes as demonstrated by GFAP (**G–I**). Eight weeks post-transplantation. β-Galactosidase-labeled HB1.F3 human neural stem cells in the lesion sites did not react with pan-*myc* antibody, indicating that *myc* oncogene in F3 neural stem cells are not expressed any longer following brain transplantation (**J–L, M–O**). Eight weeks post-transplantation. Scale bars = 50 μm. Abbreviations: β-gal, β-galactosidase; GFAP, glial fibrillary acidic protein; MAP, mitogen activated protein; NF, neurofilament.

from implanted v-*myc*-propagated human NSCs [5, 32]. In newborn mice, following intraventricular transplantation, F3 human NSCs integrated well within the subventricular layer, and from this region an extensive migration of β-gal-positive F3 cells was found in neighboring anatomical sites such as hippocampus and neostriatum at 15 weeks after transplantation, and no neoplasm formation was evident (data not shown).

DISCUSSION

In the present study, HB1.F3 human NSCs transplanted into the brain of mouse model of ICH stroke were found to survive, differentiate into neurons and glial cells, and promote behavioral improvement in ICH animals. These results indicate that the human NSCs are a valuable source of cells for stem cell-based therapy for the treatment of ICH patients.

HB1.F3, a stable clonal cell line of human NSC, has been generated from the human fetal telencephalon cells using a retroviral vector encoding v-*myc* and expresses ABCG2 [33], Musashi-1 [34], and nestin [35], cell type-specific markers for NSCs, as our previous studies have described [8, 19, 25]. When F3 NSCs are grown in serum, they differentiate into neurons and astrocytes, as shown in Figures 1 and 2, and our previous electrophysiological study has also demonstrated that F3 NSCs generate inward currents of voltage-activated sodium channels, which indicates that the neuronally differentiated F3 cells have electrophysiological characteristics of mature neurons [18]. Stable clonal cell lines of human NSCs are important and useful for three reasons: first, they provide human cells which are exceedingly difficult to obtain; second, they are easy to maintain and expand and provide a homogeneous population of cells by which identification and characterization of molecules ranging from neurotrophic factors to specific receptors and the regulatory pathways can be readily investigated; and third, human NSC lines should provide a ready and valuable source of human cells to be implanted into defined regions of nervous system to correct specific defects and eventual restoration of neurological function for various neurological disorders [5, 8, 36].

Previous studies have indicated that the injured brain and spinal cord are receptive to externally transplanted NSCs, inducing survival and differentiation of these cells and implanted NSCs preferentially migrate to areas of pathology [14–16, 36–39]. Recently, NSCs have been transplanted in the damaged brain or spinal cord for replacement of damaged neurons and for delivery of therapeutic molecules to provide neuroprotection of damaged neurons [5, 8, 14–16, 19–22, 39–44].

In the present study, transplantation of F3 human NSCs into the cerebral cortex overlying intrastriatal hemorrhagic lesion showed that a large number of F3 cells survive, migrate along the boundary zones adjacent to the hemorrhage core, and differentiate into neurons and astrocytes. These observations are consistent with previous studies that found that grafted F3 NSCs could differentiate into neurons and astrocytes in the microenvironment of lesioned brain in rats with ischemia or ICH models following i.v. injection [14–16]. It is evident that the neurogenesis and migration of transplanted NSCs are influenced by the cues or signals in the microenvironment of damaged brain as described above. Previous studies have shown that a limited degree of endogenous neurogenesis occurs in damaged region, which was induced by stroke [45, 46]. Further studies are needed to determine whether grafted F3 NSCs could integrate among the neurons of host brain, make new synaptic connections with them, and replace lost neurons. Electrophysiological studies undergoing in our laboratory in brain slices obtained from rat brain grafted with F3 NSCs so far did not yield positive evidence of new synaptic connection. Two to 8 weeks after implantation, LacZ-positive F3 NSCs migrated selectively to the hemorrhage core and also located on the border of the lesion (Fig. 4A–4E). In newborn mice, following transplantation into lateral ventricles, an extensive migration of LacZ-positive F3 NSCs was demonstrated streaking out from the lateral ventricle, the site of implantation, to distant anatomical sites including striatum, hippocampus, and olfactory bulb along the path of rostral migratory stream (unpublished data). These results suggest that when F3 cells are implanted into ventricular space, their migratory and differentiation potentials are strongly influenced by the environment signals produced by host's neighboring brain sites.

Although the mechanism by which NSCs migrate extensively in a selective manner to the pathological lesions is unclear, it is suggested that the transplanted F3 NSCs are recruited by certain chemoattractant signals produced at CNS injury sites such as cytokines, stromal cell derived factor-1 (SDF-1) [47], VEGF [32], or stem cell factor (SCF) [48]. Other cytokines with important functions in CNS development including bFGF, EGF, and TGFα are also shown to increase ischemia-induced proliferation and migration of neural progenitor cells [49]. Because F3 NSCs express CXCR4, the receptor for SDF; VEGFR1 and VEGFR2, the receptors for VEGF; and c-kit, the receptor for SDF-1 (Fig. 1C), the pathways involving SDF/CXCR4, VEGF/VEGFR, and SCF-1/c-kit are involved in the migration of F3 NSCs to the sites of ICH brain damage and also to corpus callous and hippocampus. Migration of NSCs toward sites of brain injury may represent an adaptive response of NSCs for the purpose of limiting tissue injury or repair the tissue damage.

When NSCs are implanted into different areas of the developing nervous system, they generate progeny that would normally be generated in that area at the time the cells are grafted. Thus, the same precursor cells can generate Purkinje cells when implanted into the cerebellum [50] and hippocampal neurons when introduced into the hippocampus at the time that these cells are being generated from endogenous precursors [51]. Likewise, multipotent NSCs introduced into the brain of myelin-deficient mouse could generate oligodendrocytes [52]. These observations indicate that the fate of multipotent NSCs is influenced by molecules generated from the environment into which they were placed. It seems that F3 human NSCs could also be influenced by signals produced in the CNS anatomical sites where they were implanted and terminally differentiate into CNS neurons or glial cells. The spectacular property of the immortalized human NSCs to differentiate in situ in the recipient animal brain may eventually allow targeted introduction of NSCs into defined regions of brain to correct specific defects and eventual restoration of neurological function for many types of neurological diseases with a widespread pathology. Endogenous or exogenously injected neurotrophic factors including BDNF, GDNF, CNTF, and NT-3 had a neuroprotective effect in damaged brain, including animal models of stroke [53–55].

Our RT-PCR studies show that F3 human NSCs express BDNF, GDNF, CNTF, bFGF, VEGF, HGF, and IGF that may work as neuroprotective factors. Enzyme-linked immunosorbent assay quantitations of supernatants collected from cultures of F3 human NSCs indicated that significant amounts of NGF and BDNF are secreted by the F3 human NSCs in vitro. Our preliminary study has shown that the intrastriatal injection of F3 NSCs transfected with the *BDNF* gene resulted in improved function and reduced brain damage in a rat stroke model of ICH (unpublished data). In the present study, grafted F3 NSCs located in hemorrhagic lesion sites express strong activity for NGF and BDNF, which indicate that the grafted F3 human NSCs secrete neurotrophic factors NGF and BDNF in the microenvironment of ICH and promote functional recovery.

Cell therapy has become a most promising strategy for the treatment of many human diseases, including neurological disorders. The objective of cell therapy is to replace lost cells and restore the function of damaged cells/tissues following diseases or trauma. Transplantation of renewable, homogenous, multipotent, and well-characterized NSCs into the damaged nervous system tissues should replace lost cells and restore damaged function. Previous studies have shown that the F3 human NSCs injected intravenously or transplanted in cerebrum significantly reduced motor and neurological deficits in animal models of Parkinson's disease [19], Huntington's disease [20, 21], brain ischemia/ICH [14–16],

and lysosomal storage disease [22]. The stable immortalized human NSC lines described here can be expanded readily and provide a renewable and homogeneous population of neuronal and glial cells, and they will provide most valuable means for future studies of fundamental questions in developmental neurobiology, cell and gene therapies, and research and development of new drugs and new treatment.

In conclusion, F3 human NSC line can be induced to differentiate into neurons and glial cells in vitro and in vivo and has a potential to produce several neuroprotective factors, including NGF and BDNF. The present study demonstrates that F3 human NSC cell line is not only a useful tool for the studies of neurogenesis but also as a renewable cell source for the stem cell-based cell therapy in the CNS diseases.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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